

## CYCLOHEXIMIDE INDUCED IN VIVO MODIFICATION OF ORNITHINE DECARBOXYLASE IN *PHYSARUM POLYCEPHALUM*

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Received 25 October 1974

### 1. Introduction

Ornithine decarboxylase (EC 4.1.1.17) is thought to be the rate-limiting enzyme in the pathway for the synthesis of the polyamines, putrescine, spermidine and spermine, which are essential for cell proliferation [1,2]. In the presence of inhibitors of protein or RNA synthesis, the activity of this enzyme decays with a half-life of 10–60 min [3–5], which has led to the conclusion that the sensitive response of this enzyme activity to growth conditions is associated with its high rate of synthesis and degradation. However this instability has never been shown directly, e.g. by immunological precipitation techniques. There are many conflicting reports on the half-life of this enzyme and its variation in relation to changes in enzyme activity [3–9], suggesting a more involved mechanism for the regulation of this enzyme. Since the control of polyamine levels has been shown to be important in cystic fibrosis [8], cancerous growth [6,9,11], and chemotherapy [12,13], it is essential that the mode of regulation of this important enzyme be clarified. Here we report that the rapid decay in the ornithine decarboxylase activity of *Physarum polycephalum* [3] is associated with a reversible modification of the enzyme. Within 100 min of the inhibition of protein synthesis by cycloheximide, the apparent  $K_m$  of the pyridoxal-5'-phosphate (PLP) activation of this enzyme varied seven-fold, while the  $K_m$  for the substrate, ornithine, was unchanged. We believe this modification, and not enzyme protein instability, is responsible for the rapid fluctuations in this enzyme's activity associated with variations in cell growth.

### 2. Materials and methods

Pyridoxal-5'-phosphate, L-ornithine and cycloheximide were purchased from Sigma Chemical Co. DL-[1- $^{14}$ C] Ornithine · HCl (29  $\mu$ Ci/mole) was obtained from Amersham/Searle Corporation.

#### 2.1. Culture techniques

Cultures of *P. polycephalum* were maintained and samples extracted as described earlier [3].

#### 2.2. Preparation of enzyme extracts

Frozen tissue pellets containing about 3–5 mg of protein were suspended in 3 ml of 0.05 M borate buffer (pH 8.15), containing 0.5 mM dithiothreitol and 0.2 mM EDTA, disrupted by sonicating for 30 sec and assayed immediately.

#### 2.3. Ornithine decarboxylase activity

Enzyme activity was determined by measuring the liberation of  $^{14}$ CO<sub>2</sub> from DL-[1- $^{14}$ C]ornithine as detailed earlier [3], except hyamine hydroxide was used to absorb the  $^{14}$ CO<sub>2</sub> which was counted in a toluene-based scintillation fluid. The incubation mixtures are described in the figure legends.

### 3. Results

#### 3.1. Effects of cycloheximide on ornithine decarboxylase activity

The inhibition of protein synthesis in *P. polycephalum* by 25  $\mu$ g/ml cycloheximide induces a very rapid loss in ornithine decarboxylase activity when

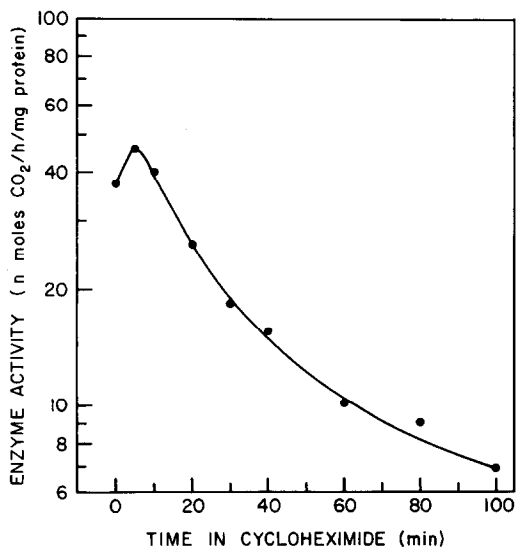


Fig. 1. Decay in ornithine decarboxylase activity in microplasmidia exposed to 25  $\mu\text{g/ml}$  of cycloheximide. Inhibitor was added to a shake-flask culture of *Physarum* microplasmidia at time 0 and subsequently 3 ml samples were extracted, frozen and eventually assayed as described in Materials and methods. Assay mix contained 100  $\mu\text{l}$  of enzyme extract and 1.9 ml of 0.05 M borate buffer (pH 8.15), 0.5 mM dithiothreitol, 0.2 mM EDTA, 0.05 mM L-ornithine (0.05  $\mu\text{Ci}$ ), and 1  $\mu\text{M}$  PLP.

assayed as described in the legend to fig. 1. If the temporary increase in activity during the first few minutes of the cycloheximide inhibition is ignored, the apparent half-life calculated between 5 and 40 min of treatment is about 18 min, comparable to the short half-lives reported in mammalian tissues [4–9].

### 3.2. Effect of cycloheximide on substrate stimulation of ornithine decarboxylase activity

To investigate the possibility that an alteration in the enzyme's ability to bind substrate may be partially responsible for the rapid variation in activity in response to cycloheximide, several of the above fractions were assayed for activity in various ornithine concentrations. The double reciprocal plots of these data (fig. 2) indicate that the apparent  $K_m$  of ornithine activation is unaffected by the cycloheximide treatment, with an average value of 0.27 mM.

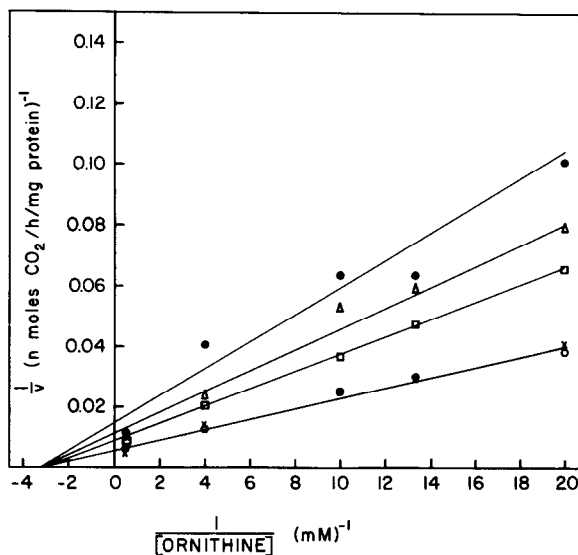


Fig. 2. Substrate stimulation of ornithine decarboxylase during treatment with cycloheximide. The enzyme samples representing 0 ( $\circ$ — $\circ$ — $\circ$ ), 5 (X—X—X), 20 ( $\square$ — $\square$ — $\square$ ), 60 ( $\triangle$ — $\triangle$ — $\triangle$ ) and 100 ( $\bullet$ — $\bullet$ — $\bullet$ ) min exposure to cycloheximide of fig. 1 were assayed at varying ornithine concentrations, using 50  $\mu\text{M}$  PLP in the assay mix and procedure outlined in fig. 1. Lines were drawn by a simple linear regression.

### 3.3. Effect of cycloheximide on coenzyme stimulation of ornithine decarboxylase

Since there is no detectable ornithine decarboxylase activity in crude enzyme preparations without added PLP [3], we also tested the ability of this coenzyme to stimulate activity as a possible indication of an altered enzyme state. Each of the nine enzyme fractions from the experiment illustrated in fig. 1 was assayed for activity in the presence of varying concentrations of PLP. The double reciprocal plot of this data (fig. 3) illustrates the extreme variation in PLP binding ability resulting from the inhibition of protein synthesis. The apparent  $K_m$ 's for PLP activation were extrapolated from this data using a simple linear regression program, and plotted with respect to time in cycloheximide (fig. 4). This plot illustrates a sharp initial decrease and an eventual seven-fold increase in the  $K_m$  of PLP activation. Since any change in this  $K_m$  would cause an inverse variation

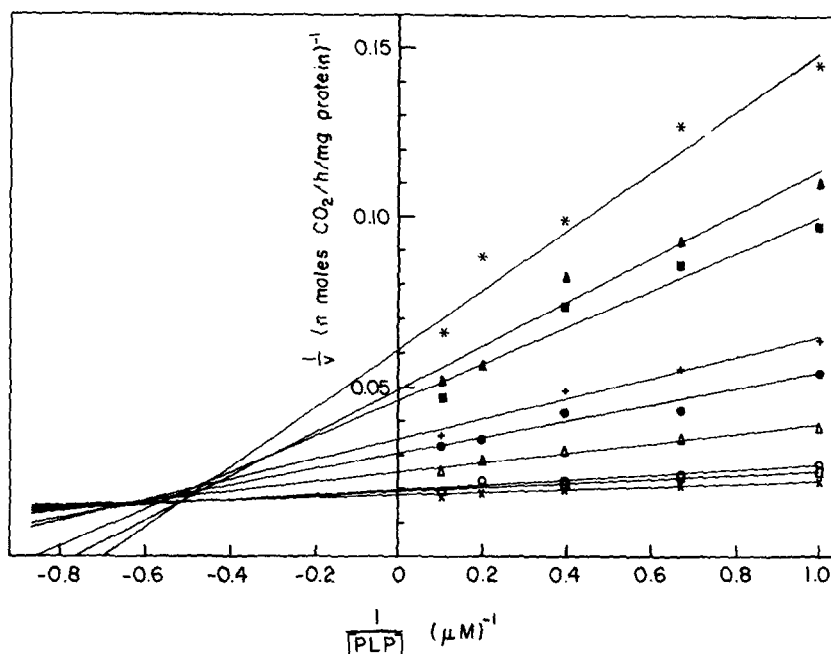
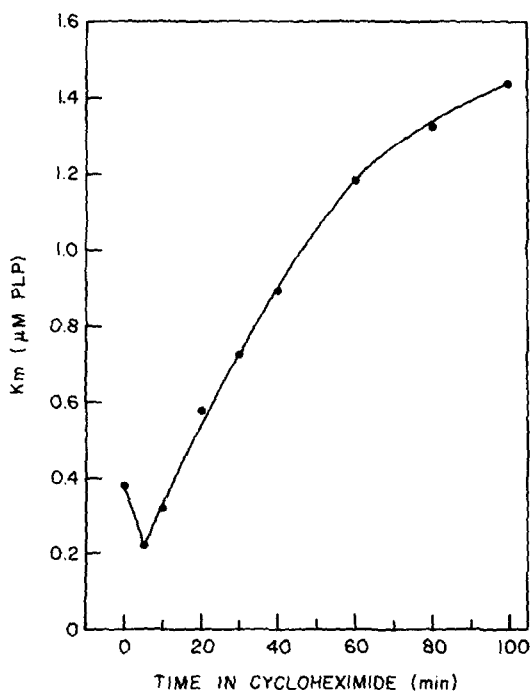


Fig. 3. Variation in the stimulation of ornithine decarboxylase activity by PLP during inhibition with cycloheximide. The enzyme samples of fig. 1 at 0 (○-○-○), 5 (×-×-×), 10 (□-□-□), 20 (△-△-△), 30 (●-●-●), 40 (+-+-+), 60 (■-■-■), 80 (▲-▲-▲) and 100 (\*-\*\*) min were assayed at varying PLP concentrations using the techniques and basic assay mix described in the legend to fig. 1. Lines were drawn by a simple linear regression.



in activity assayed in coenzyme levels that are less saturating, it is obvious that this alteration in enzyme character is at least partially responsible for the rapid variations in enzyme activity noted in fig. 1.

To illustrate the extent this enzyme alteration actually affected the pattern in fig. 1 the enzyme activity of each sample was calculated at saturating PLP concentrations using the extrapolated  $y$ -intercepts of fig. 3. When the logs of these maximum velocities are plotted against time (fig. 5) the characteristic pattern of a sharp activity increase and rapid decay is much less pronounced, although still obvious. An optically fitted exponential decay curve drawn from these points indicates a  $T_{1/2}$  of 70 min,

Fig. 4. Changes in the  $K_m$  of PLP activation of ornithine decarboxylase activity during cycloheximide inhibition. The negative inverse of the extrapolated  $x$ -intercepts of fig. 3 are plotted to express  $K_m$  variation during cycloheximide treatment.

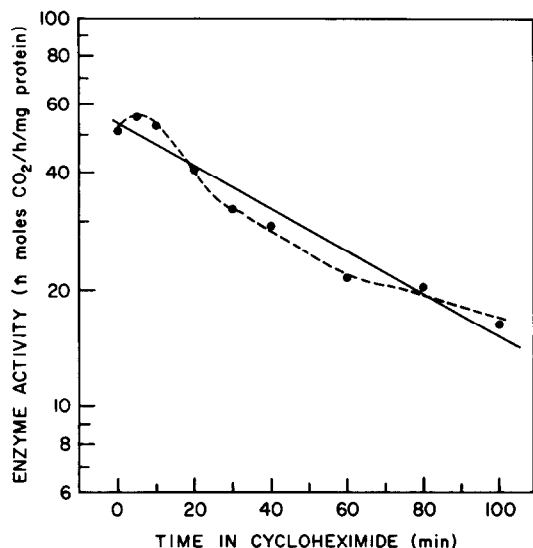


Fig. 5. Cycloheximide induced decrease in enzyme activity at saturating coenzyme levels. The inverse of the y-intercepts of the data of fig. 3, representing the extrapolated reaction velocities at saturating PLP concentrations, are plotted with respect to time on cycloheximide. The curve connecting data points (---) is seen to diverge from a logarithmic decay curve (—) in a pattern similar to the more pronounced variations seen at limiting PLP concentrations in fig. 1.

considerably longer than the 18 min calculated from fig. 1.

### 3.4. Stability of the enzyme alteration produced by cycloheximide inhibition

To test the possibility that a small molecule may be present in the crude enzyme extract which inhibits the coenzyme activation of this enzyme, 1 ml samples of the crude enzyme preparation from 0 and 100 min after cycloheximide treatment were each dialyzed overnight against two changes of 400 ml buffer at 4°C, and their PLP stimulation kinetics assayed as before. In each case the dialyzed sample had the same apparent PLP  $K_m$  fractions were mixed and assayed to test for the presence of a soluble regulatory molecule in the crude enzyme extracts. In the PLP concentration range tested from 0.5 to 10  $\mu$ M, the activity of the mix did not deviate from that expected from the sum of the two enzyme sub-fractions.

## 4. Discussion

The results presented in this paper suggest a rapid modification in ornithine decarboxylase caused by the inhibition of protein synthesis. Since this modification involves a marked increase in the enzyme's apparent  $K_m$  for PLP, then assays performed at limiting PLP concentrations reflect these rapid enzyme alterations as apparent variations in the enzyme activity (fig. 1). Heretofore, possible variations in the coenzyme stimulation of this enzyme have not been investigated, yet limiting coenzyme concentrations are indicated in the majority of assay procedures reported in the literature, because these involve either Tris buffer or great excesses of ornithine, both of which will readily form Schiff-bases with the coenzyme and lower its effective concentrations [14,15]. We believe, therefore, that it is basically this enzyme modification, and not the innate instability of the enzyme molecule, which is responsible for the pattern of rapid enzyme activity variations reported for this enzyme.

If inhibition of protein synthesis merely modified the ability of ornithine decarboxylase to be activated by PLP, then assays in the presence of saturating levels of PLP would be unaffected by this modification and thus could be used to indicate the exponential decay of this enzyme (half-life). The extrapolated enzyme velocities at saturating PLP levels (fig. 5), however, deviate from the exponential decrease in activity predicted and they appear to lose activity in a pattern which resembles the inhibition due to the alteration in the  $K_m$  for PLP. This suggests that the rapid change in ornithine decarboxylase activity following this inhibition of protein synthesis may result from an enzyme modification which affects both the turnover number, or efficiency, of the enzyme and its ability to bind the coenzyme. If this modification does affect both these enzyme functions, as is the case with some allosteric regulators, then the actual half-life of this enzyme is probably much longer than the 70 min calculated from fig. 5.

Although this paper reports only on the enzyme modification in response to cycloheximide, we have found that similar modifications may be induced by culture starvation and the inhibitors of RNA synthesis, cordycepin and actinomycin D. This would suggest that a small protein factor must be continually

synthesized to maintain the optimal activity of the enzyme or else a negative regulatory molecule, such as the idling factor in bacteria, is produced when RNAs and proteins are not being synthesized. Such a regulatory molecule has already been implicated in the repressive enzyme modifications of bacterial ornithine decarboxylase [16].

The stimulation of the enzyme activity during the first 5 min of cycloheximide treatment is due to an increased  $V_{\max}$  and a decreased  $K_m$  for PLP, suggesting that the modification of this enzyme is reversible. Thus rapid increases in enzyme activity which accompany the stimulation of growth, may reflect the reversal of an inhibitory modification rather than the stimulation of new enzyme synthesis. Recent studies in our laboratory indicate that the PLP  $K_m$  of this enzyme fluctuates over a 10-fold range in response to variations in developmental state or the phase of the mitotic cycle, indicating that this modification may be the natural mechanism for regulation of this critical enzyme pathway in the low PLP concentrations found in vivo.

### Acknowledgements

We wish to thank Terry Gawel for her technical assistance. J.L.A.M. was supported by a Dean's Fund Summer Faculty Research Grant from Northern Illinois University. This research was supported by

grant #74-11 to J.L.A.M. from the Illinois Division of the American Cancer Society.

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